

NF-kappa B Protein Purification from Bovine Spleen: Nucleotide Stimulation and Binding Site Specificity

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Notes:

NF- κ B protein purification from bovine spleen: Nucleotide stimulation and binding site specificity

(κ immunoglobulin light chain gene/enhancer/interleukin 2/human immunodeficiency virus)

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ABSTRACT The activity of the enhancer for the κ immunoglobulin light chain gene critically depends on the presence in the nucleus of the NF- κ B protein. We purified NF- κ B over 50,000-fold and identified two protein species, 42 and 44 kDa, that could be eluted and renatured from a sodium dodecyl sulfate/polyacrylamide gel to give specific DNA-binding activity. Binding of the purified bovine NF- κ B as well as that from human and murine B- or T-lymphoid cell extracts was dramatically stimulated by nucleoside triphosphates. This effect distinguished NF- κ B from a related factor, H2-TF1. Purified NF- κ B interacted efficiently with regulatory sequences that function during either B- or T-lymphocyte activation, including the human immunodeficiency virus enhancer and a NF- κ B binding site we detected in the interleukin 2 enhancer.

The differentiation of B lymphocytes is a well-characterized example of changes in gene activity during progression through a cell lineage (1). One important event is expression of the immunoglobulin light chain gene. NF- κ B, a DNA-binding protein that recognizes a 10-base-pair (bp) enhancer sequence, is crucial for expression of the κ light chain gene (2–6). Only mature B cells and exceptional T cell lines express NF- κ B binding constitutively (2), but binding can be induced in other cells by agents such as lipopolysaccharide (LPS), cycloheximide, and phorbol esters (3).

Recently, an NF- κ B-like factor was shown to play an important role in the expression of the interleukin 2 (IL-2) receptor α chain during activation of T lymphocytes (7–9). The enhancer of human immunodeficiency virus (HIV)-1, a T-lymphotropic virus, also binds an NF- κ B-like factor (10–14). These results may indicate that NF- κ B participates in expression of genes during activation of both B and T lymphocytes. Alternatively, there is evidence indicating that a distinct factor with some characteristics of NF- κ B may exist in T cells (13).

A different nuclear factor, H2-TF1, has a binding site with close sequence similarity to that of NF- κ B. H2-TF1 is present in lymphoid and nonlymphoid cells and plays a role in major histocompatibility complex (MHC) class I gene expression (15, 16). Recently, a clone was selected by the H2-TF1 binding site from a λ gt11 expression library (17). The binding characteristics of this clone led to the proposal that H2-TF1 may be a constitutively active form of NF- κ B (16, 17). Thus, it is important to compare biochemical features of these binding proteins to determine their relationship.

We purified NF- κ B over 50,000-fold to a set of two protein species of 42 and 44 kDa. Nucleoside triphosphates were found to dramatically increase the binding of purified NF- κ B as well as unpurified binding protein from human and mouse lymphoid cell extracts. By contrast, H2-TF1 was not stimulated by nucleoside triphosphates. Highly purified NF- κ B

interacted with regulatory sequences expressed during either B- or T-lymphocyte activation. The most efficient binding we detected was to a site in the IL-2 gene enhancer. Thus the same NF- κ B protein is likely to regulate genes during activation of both B and T lymphocytes.

MATERIALS AND METHODS

Preparation of Nuclear Extracts. Fresh bovine spleen was pressed through a 60-mesh screen and erythrocytes were lysed in 4 vol of 0.83% NH₄Cl, pH 7.2. Nuclear extracts were prepared at 4°C according to Dignam *et al.* (18). All buffers included leupeptin at 0.3 μ g/ml, 5 mM phenylmethylsulfonyl fluoride, and antipain at 0.3 μ g/ml. Typically, 1.5 kg of spleen yielded 3.6 g of crude nuclear extract. Protein was measured by Bradford assay (19).

Chromatographic Fractionation. Sephacryl S-300 (Pharmacia) (1 liter in a 5 \times 60 cm column) was equilibrated with buffer D [20 mM Hepes, pH 7.9/0.2 mM EDTA/0.5 mM dithiothreitol/0.1 M KCl/20% (vol/vol) glycerol]. Portions (190 ml) of nuclear extract (600 mg of protein) were precipitated with 60 g of ammonium sulfate (50% saturation), resuspended in 50 ml of buffer D, and fractionated at 0.06 column volumes per hour. Washed phosphocellulose (250 ml in a 5 \times 25 cm column) was equilibrated with buffer D. The Sephacryl S-300 active fraction (750 ml; 200 mg of protein) was applied, washed with 1250 ml of buffer D, and eluted with a 1-liter gradient from 0.1 to 1.0 M KCl in buffer D. Hydroxylapatite chromatography using a Bio-Gel 100 \times 7.5 mm HPHT column (Bio-Rad) was previously described (20). The phosphocellulose peak was dialyzed against buffer A (50 mM KH₂PO₄, pH 7.5/1 mM dithiothreitol/0.01% Nonidet P-40/0.1 mM phenylmethylsulfonyl fluoride/0.01 mM CaCl₂/10% glycerol), applied to the HPHT column at a flow rate of 0.5 ml/min, washed with 22.5 ml of buffer A, and eluted with a 15-ml gradient from 0 to 1 M NaCl in buffer A.

DNA affinity chromatography was performed according to Kadonaga and Tjian (21), using an oligonucleotide containing 22 base pairs from the κ enhancer (5'-CAGAGGGGACTT-TCCGAGAGGC-3') with TCGA 5' overhanging ends. The column contained 1.2 nmol of binding sites per ml of Sepharose gel. Chromatography was carried out in buffer Q (20 mM Hepes, pH 7.9/100 mM NaCl/0.2 mM EDTA/0.5 mM dithiothreitol/0.05% Nonidet P-40/20% glycerol). The load was mixed with poly(dI-dC) (Pharmacia) at 25 μ g/mg on ice for 15 min and applied dropwise to the column. The column was washed with 10 column volumes of buffer Q and step eluted with buffer Q containing successively 0.1 M, 0.2 M, 0.3 M, 0.5 M, 0.75 M, 1.0 M, and 2.0 M NaCl. Activity eluted in the 0.5 M NaCl fraction. When the *Escherichia coli* DNA column was used, buffer Q/0.1 M KCl was the loading buffer and the protein was eluted with a 0.1 to 1.0 M NaCl

gradient in buffer Q. Fractions were analyzed by electrophoresis on miniature NaDodSO₄/polyacrylamide gels (22) and silver staining (23).

Binding Assays. Mobility shift electrophoresis assay mixtures (5) contained 0.25 ng of ³²P-labeled DNA fragment (15,000 cpm), 10 mM Tris-HCl at pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl₂, 3 mM GTP (omitted in experiments in which the amount of added GTP was varied), and 5% glycerol. Assays of crude fractions used 0.5 μg of poly(dI-dC) and those of affinity fractions used no poly(dI-dC) but included 1.25 μg of bovine serum albumin. The IL-2 receptor α-chain probe was generously provided by S. Cross and W. Leonard (National Institutes of Health), and the MHC class I probe (17) was generously provided by H. Singh (Massachusetts Institute of Technology). Competition assays used the κ enhancer oligonucleotide described above or an oligonucleotide from nucleotides -186 to -210 of the human IL-2 promoter (5'-CAAAGAGGGATTTCACCTACATCC-3') (24). Strands were annealed and diluted in 20 mM Tris-HCl, pH 7.6/100 mM NaCl. Methylation interference analysis (2) and denaturation-renaturation analysis (25) were performed as described previously.

DNase I footprinting by the procedure of Jones *et al.* (26) used either wild-type or mutant versions (7) of a 150-bp *Ava* I-*Bgl* II HXBH2 long terminal repeat (LTR) fragment (provided by Mark Feinberg, Whitehead Institute) radioactively labeled at the *Ava* I site.

RESULTS

Extracts from Bovine Spleen Cells Contain NF-κB. Bovine spleen nuclear extracts were found to have levels of a binding activity comparable to those of NF-κB in WEHI 231 murine B lymphoid tumor cells (Fig. 1, lanes 1 and 2). Interestingly, the bovine nucleoprotein complex formed with sites from the MHC class I gene promoter, the HIV-1 LTR, and the IL-2 receptor α-chain promoter (see Fig. 2B and Fig. 5). Mutations that abrogate murine NF-κB binding also eliminated the bovine complex (Fig. 1, compare lane 3 to lane 4 and lane 5 to lane 6). The complex was not found in bovine brain, thymus, or a kidney cell line but could be induced by phorbol esters (C. Murre, M.J.L., and D.B., unpublished results).

We next examined the close contacts between protein and DNA by comparing bovine extracts to those from LPS- and cycloheximide-treated murine 70Z/3 cells in a methylation interference assay (2, 3). Methylation of three guanines that interfered with formation of the 70Z/3 complex also partially or totally interfered with formation of the bovine complex (Fig. 2A, lanes 2 and 4). On the opposite strand methylation of three guanines interfered with formation of both the

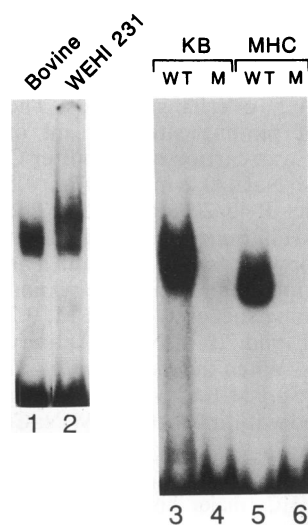


FIG. 1. Mobility shift electrophoresis assay of bovine spleen nuclear extracts. Ten micrograms of protein from either crude bovine nuclear extracts (lanes 1 and 3-6) or WEHI 231 murine B cell nuclear extracts (lane 2) was used with either wild-type (WT) (lanes 1, 2, and 3) or mutant (M) (lane 4) versions of the κ enhancer binding site (KB) (5) or with the wild-type (WT, lane 5) or mutant (M, lane 6) of the MHC class I binding site (MHC) (17).

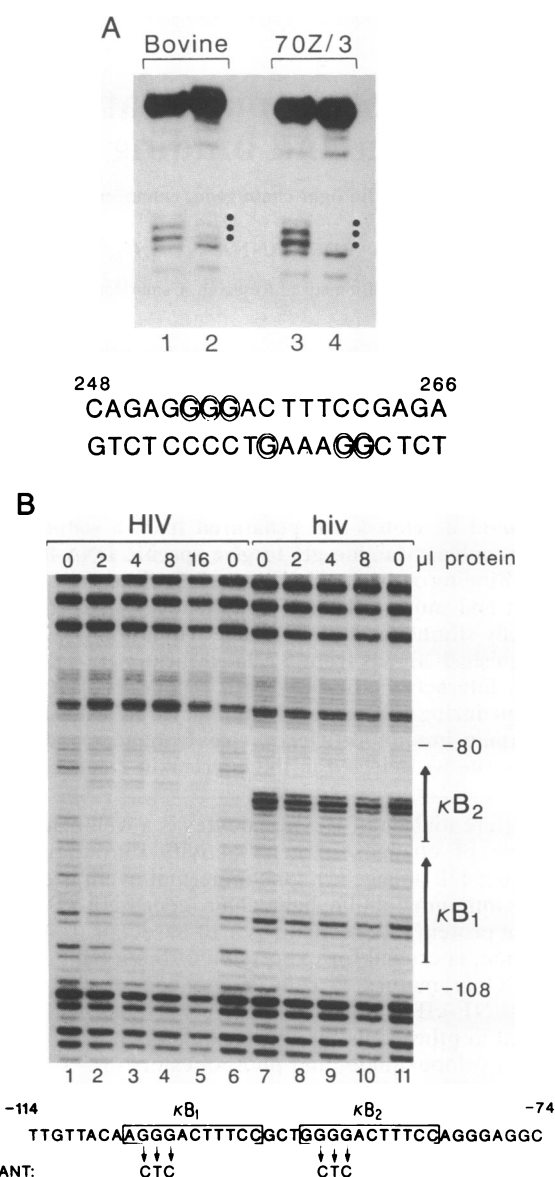


FIG. 2. Methylation interference and DNase I footprinting analyses. (A) Methylation interference assay using the κ enhancer site compares bovine extract to 70Z/3 murine pre-B lymphocytes stimulated with *E. coli* strain O55:B5 LPS (Difco) and cycloheximide (2, 3). Lanes 1 and 3 are free probe and lanes 2 and 4 are probe from the nucleoprotein complex. Dots indicate guanine residues whose methylation interferes with complex formation. Below is the κ enhancer site with circles around the guanine residues that interfere with complex formation when methylated. (B) DNase I footprint with wild-type (HIV) or mutant (hiv) HIV-1 enhancer sequences. Indicated is the position of the two NF-κB binding motifs and the limits of the protected region (-80 to -108). Below is shown the HIV-1 enhancer region with the NF-κB recognition motifs boxed and arrows indicating the base changes in the mutant sequence (10).

murine and bovine complexes (data not shown). The bottom of Fig. 2A summarizes the interference pattern. Because of its specific binding, tissue distribution, phorbol ester inducibility, and other biochemical properties described below, we conclude that the identified factor is the bovine homologue of the murine or human NF-κB protein.

Chromatographic Purification of the NF-κB Binding Protein. Crude nuclear extract was fractionated by Sephacryl S-300 chromatography (see Table 1). The active pool was applied to a P-11 phosphocellulose column and eluted with a linear KCl gradient from 0.1 to 1.0 M. Binding activity eluted between 0.4 and 0.6 M KCl. To eliminate contaminants that

Table 1. Purification of NF- κ B

Step	Fraction	Protein, mg	Volume, ml	Total activity, units	Specific activity, units/mg	Overall purification, -fold	Overall yield, %
I	Nuclear extract	3600	1150	115,000	32	—	100
II	Sephacryl S-300	690	2170	41,000	60	1.9	36
III	Phosphocellulose	45	640	37,000	820	26	32
IV	Hydroxylapatite	6.2	177	52,000	8,300	260	45
V	<i>E. coli</i> DNA	0.9	105	24,000	27,000	840	21
VI	NF- κ B affinity	0.005	1.0	9,500	1,900,000	59,000	8.3

Activity is given as a relative binding unit, with 1 unit being equivalent to the binding detected in 10 μ l of the crude nuclear extract.

bound to DNA at high salt concentrations, hydroxylapatite chromatography was used; the activity eluted sharply between 0.5 and 0.6 M NaCl. Total activity increased, apparently because this step removed an inhibitor of binding. Finally, a site-specific DNA affinity column using NF- κ B binding sequences from the κ enhancer was used (21). In some preparations, this step was preceded by a nonspecific *E. coli* DNA-Sepharose column. Alternatively, nonspecific competitor DNA was added to the load. Either procedure provided an additional purification of 50- to 100-fold. A nearly 60,000-fold purification was obtained with an 8% recovery (Table 1). Because the yield was 5 μ g of protein from approximately 4×10^{11} spleen cells (after elimination of erythrocytes), we estimate there are 1500 molecules of NF- κ B per cell.* This suggests NF- κ B is a low-abundance protein in bovine spleen.

Chromatographic fractions were compared by NaDodSO₄/polyacrylamide gel electrophoresis and silver staining. Early steps yielded complex mixtures of polypeptides (Fig. 3A, lanes 1–5). By contrast, only three polypeptides, 42, 44, and 55 kDa, were detected in active affinity fractions (Fig. 3A, compare lane 7 to lanes 6 and 8). Because the 55-kDa species was also found in an inactive fraction (Fig. 3A, lane 8), it is likely that either the 42- or the 44-kDa species or both are responsible for binding.

To establish that the purified proteins contained binding activity, we excised slices from a preparative NaDodSO₄/

polyacrylamide gel (shown in Fig. 3A) and eluted the protein. Eluted material was then denatured, renatured, and assayed for binding by mobility shift gel electrophoresis. Only the gel slice containing the 42- and 44-kDa proteins yielded binding activity; that containing the 55-kDa protein and other slices did not (Fig. 3B). The renatured complex comigrated with NF- κ B and did not bind the mutant probe, demonstrating that the 42- and 44-kDa proteins have specific NF- κ B binding activity. These results were consistent with the molecular weight determination of bovine NF- κ B in crude extracts and with the analysis of partially purified fractions by two-dimensional electrophoresis (P. Baeuerle, M.J.L., and D.B., unpublished results). Glycerol gradient sedimentation suggested that purified bovine NF- κ B exists in solution as a dimer (data not shown).

Nucleoside Triphosphates Stimulate Binding of NF- κ B *in Vitro*. In testing possible cofactors for binding, we found that guanosine 5'-triphosphate (GTP) powerfully stimulated the binding of purified NF- κ B. At 3 mM, GTP gave up to a 100-fold stimulation (Fig. 4A, top) but greater amounts decreased binding. The triphosphate was required, since GDP, GMP, cGMP, and guanosine 5'-[β , γ -imido]triphosphate gave little or no effect (Fig. 4A, bottom). Either ATP or GTP could stimulate binding of NF- κ B in crude nuclear extracts (Fig. 4B). Of particular importance, CEM, a human T cell line that efficiently replicates HIV, contains a binding activity specific for NF- κ B sites that was markedly stimulated by GTP (Fig. 4B). Further experiments showed that any of the four standard deoxyribonucleoside triphosphates or the four ribonucleoside triphosphates increased binding at similar concentrations (data not shown).

*The estimate was based on 60% of the final protein yield being NF- κ B, such that $5(0.60)/0.08 = 40 \mu$ g maximum theoretical yield. This is 1×10^{-9} mol or 6×10^{14} molecules from 4×10^{11} cells or 1500 molecules per cell.

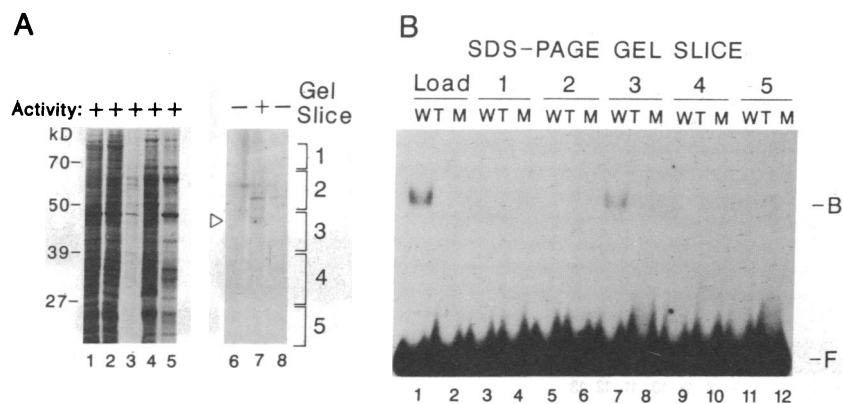


FIG. 3. PAGE analysis of chromatographic fractions. (A) NaDodSO₄ (SDS) polyacrylamide gel stained with silver. One binding unit was used of the crude nuclear extract (lane 1) and the activity peaks from the Sephacryl S-300 (lane 2) and phosphocellulose (lane 3) purified fractions. Some of the phosphocellulose sample was lost by precipitation in NaDodSO₄. Ten binding units were used of hydroxylapatite (lane 4), and *E. coli* DNA (lane 5) purified fractions. For NaCl step elutions from the affinity chromatography the 50- μ l samples are 0.2 M (lane 6), 0.5 M (\approx 450 binding units; lane 7), and 0.75 M (lane 8). At the right are the boundaries of gel slices used for denaturation-renaturation. The 42- and 44-kDa species are indicated by an arrowhead. (B) Mobility shift electrophoresis assay of renatured proteins from gel slices 1–5 as indicated in (A) or the starting material (Load). Probes are the wild-type (WT) or mutant (M) κ enhancer binding sites (5). Indicated at the right are the positions of the bound (B) and free (F) probes.

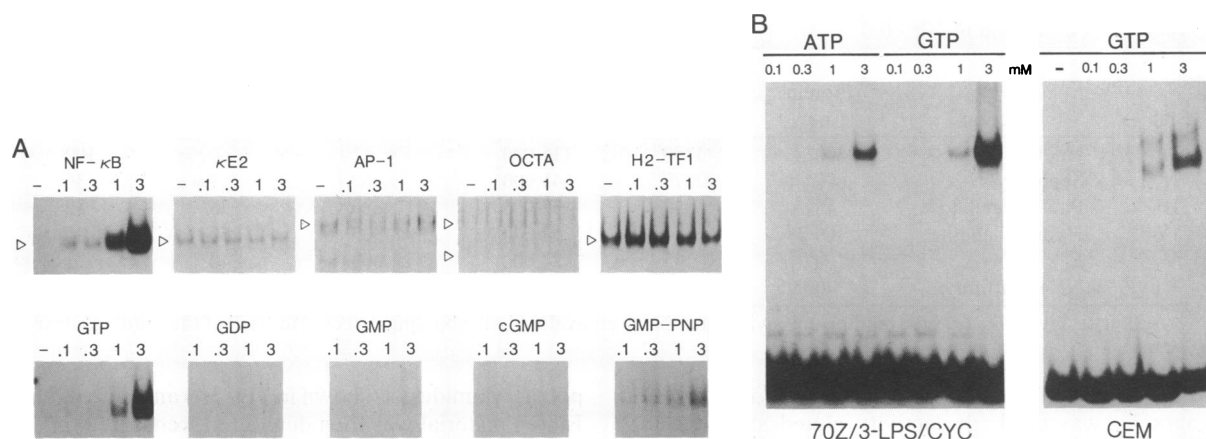


FIG. 4. (A) Nucleoside triphosphate stimulation of NF- κ B binding. Each panel shows the nucleoprotein complex formed in the presence of the millimolar amounts of GTP (top) or GTP and related compounds as indicated (bottom); the free probe is not shown. The top row shows the complex of 2 μ l of purified bovine NF- κ B with the κ enhancer probe (5) (NF- κ B); 12.5 μ g of 38B9 murine pre-B lymphoma nuclear extract with the NF- κ E2 probe (5) (κ E2); 7.5 μ g of HeLa human cervical carcinoma nuclear extract with the activator protein 1 probe (27) (AP-1); 3 μ g of WEHI-231 murine B lymphoma nuclear extract with the immunoglobulin enhancer octanucleotide probe (5) (OCTA); and 15 μ g of a mouse erythroleukemia line (MEL) nuclear extract with the H2-TF1 binding site (17) (H2-TF1). Complexes are indicated by arrowheads. The bottom row shows titration of GTP, guanosine 5'-diphosphate (GDP), guanosine 5'-monophosphate (GMP), guanosine 3',5'-cyclic monophosphate (cGMP), or guanosine 5'-[β , γ -imido]triphosphate (GMP-PNP), using 2 μ l of purified bovine NF- κ B with the κ enhancer probe (5). (B) Adenosine 5'-triphosphate (ATP) and GTP stimulation was carried out with the millimolar amount of nucleotide indicated above each lane. Crude nuclear extracts from 70Z/3 murine pre-B lymphoma stimulated with LPS and cycloheximide (70Z/3-LPS/CYC) as previously described (3) (2.25 μ g per lane) or from CEM human T cells (27) (6 μ g per lane) were used with the κ enhancer probe (5).

We next tested nucleotide stimulation of H2-TF1 binding (16, 17). No stimulation of H2-TF1 binding was seen at any concentration of GTP (Fig. 4A, top), indicating a clear biochemical difference between it and NF- κ B. In addition, several other DNA-binding factors could not be stimulated to bind (Fig. 4A, top).

Purified NF- κ B Recognizes Multiple Gene Regulatory Sequences Involved in Lymphocyte Activation. We qualitatively and quantitatively assessed the binding of the purified protein to control sequences of genes expressed during B- or T-lymphocyte activation. NF- κ B binding was qualitatively assessed by using the HIV LTR enhancer in DNase I footprinting. The footprint was identical to that observed with extracts from activated T lymphocytes and clearly different from that seen with nonlymphoid extracts (Fig. 2B; see ref. 14). To quantitatively assess binding, the interactions of NF- κ B with the κ enhancer, MHC class I promoter, and IL-2 receptor α -chain promoter sequences were tested in a

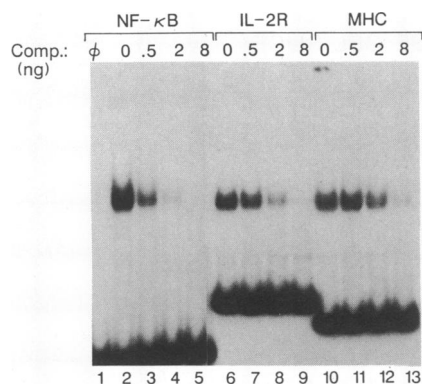


FIG. 5. Competition binding assay of NF- κ B cognate motifs from different genes. Assays used the κ enhancer probe (5) (NF- κ B, lanes 1–5), the IL-2 receptor α -chain promoter probe from a subclone of the region of –228 to –298 (28) (IL-2R, lanes 6–9), or the H2-TF1 binding site probe from the MHC class I promoter (17) (MHC, lanes 10–13). All assays included 2 μ l of purified bovine NF- κ B except lane 1 (ϕ) and the NF- κ B oligonucleotide competitor in the nanogram amounts shown above each lane.

competition assay. Each of the binding sites was used as a radiolabeled probe and a double-stranded κ enhancer oligonucleotide was added as unlabeled competitor. In each case, the competitor impaired binding at 8 ng (Fig. 5). Competition for binding to the κ enhancer was slightly more prominent at lower concentrations, suggesting that the B-cell-derived factor does not bind better but may in fact bind less well to the κ site. Nonspecific oligonucleotides had no effect (data not shown).

Since NF- κ B has been clearly implicated in the expression of the IL-2 receptor, we investigated whether NF- κ B might coordinately regulate the IL-2 gene (7–9, 29). The enhancer of the human gene contains a sequence (5'-AGGGATTTCAC-3') from position –195 to –205 that has significant homology to the NF- κ B motif (24, 29). This sequence is exactly conserved between the mouse and human genes (30),

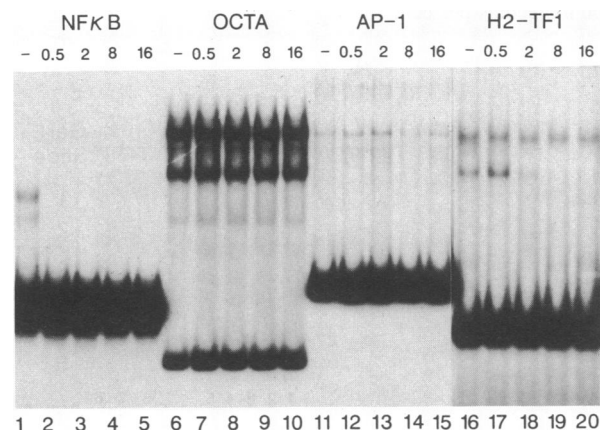


FIG. 6. Competition binding assay using sequences from the IL-2 gene promoter. Protein extracts and probes are the same as those described in the legend to Fig. 4 for purified NF- κ B (NF- κ B; lanes 1–5), the immunoglobulin enhancer octanucleotide factors (OCTA; lanes 6–10), the AP-1 factor (AP-1; lanes 11–15), and the H2-TF1 factor (H2-TF1; lanes 16–20). Annealed IL-2 oligonucleotides were added in the nanogram amounts indicated at the top; a dash indicates that only buffer was added.

and a DNase I footprint over this region was found by using extracts from activated T cells (29). We found that a double-stranded oligonucleotide containing the IL-2 site competed for NF- κ B efficiently (Fig. 6). This site was more than 4-fold better than the κ enhancer motif (compare Fig. 6 with Fig. 5). The oligonucleotide did not compete for several other DNA-binding proteins (Fig. 6). Especially important is the fact that the H2-TF1 factor did not compete, suggesting H2-TF1 has less than 1/30th the affinity for the IL-2 sequence that NF- κ B has. Interestingly, a second nucleoprotein complex with the H2-TF1 probe of faster mobility competed equivalently to NF- κ B (Fig. 6), but this has not been studied further.

DISCUSSION

The purpose of our study was to determine the biochemical features and binding specificity of purified NF- κ B. We devised a purification scheme that identified two polypeptides, 42 and 44 kDa, the smaller of which may be a proteolytic product of the larger protein. Binding of these polypeptides was dramatically stimulated *in vitro* by nucleoside triphosphates. Moreover, purified NF- κ B reproduced the DNase I footprint obtained with T-lymphoid extracts on the HIV-1 enhancer and bound tightly to regulatory sites from genes expressed in both B and T lymphocytes.

At least five factors have been identified that can bind to sequences related to the NF- κ B motif: NF- κ B, H2-TF1, KBF-1, enhancer binding protein 1 (EBP-1) and HIV enhancer binding protein 86 (HIVEN86) (2, 13, 16, 20, 31). Of these, only NF- κ B has been clearly shown to be tissue restricted (to B lymphocytes) in its constitutive expression. HIVEN86 appears to be closely related to NF- κ B in its binding specificity and inducibility in lymphoid cells. EBP-1, κ BF-1, and H2-TF1 are all found in nonlymphoid cells and may be identical. Previously, it was proposed that H2-TF1 is a constitutive form of the NF- κ B protein (16, 17). However, differences between H2-TF1 and NF- κ B were noted in both their contacts with DNA and their relative preference of binding sites (15, 16). We now extend this comparison by showing that a site in the IL-2 gene is recognized over 30-fold better by NF- κ B than by H2-TF1. Also, nucleoside triphosphates greatly stimulate NF- κ B binding but not H2-TF1 binding. The magnitudes of these differences suggest that NF- κ B and H2-TF1 arise from different gene products. A precedent in this regard is the ubiquitously expressed nuclear factor A1 (NF-A1) protein and the lymphoid-specific NF-A2 protein, which bind an octamer motif identically but are encoded by different genes (32).

The dramatic stimulation of binding by nucleotides was unique to NF- κ B among several DNA-binding proteins tested. The only other report of such a phenomenon of which we are aware is the increase in binding of simian virus 40 tumor (T) antigen to DNA caused by ATP (33). The nucleotide effect in that case was quite different in that only ATP and dATP were effective. Since nucleoside triphosphates stimulated highly purified NF- κ B, the nucleotides may directly interact with the protein. This interaction could cause a conformational change, which may increase the affinity of binding or the rate at which complex is formed. The effect depended on the nucleoside triphosphates and was not seen with the nonhydrolyzable imido analogue of GTP, indicating that hydrolysis of the nucleotide could be involved. The stimulation of NF- κ B by nucleoside triphosphates may represent another means of regulating DNA-binding activity.

The participation of the NF- κ B protein, as our data suggest, in both B- and T-cell activation is a regulatory puzzle. What specifies certain genes, such as immunoglobulin κ gene, to take part in the B-cell response and others, such as the IL-2 gene and its receptor, to be expressed during the T-cell response? Though expression of the IL-2 receptor

α chain (Tac antigen) does occur in some B-lymphocyte lines, its role in a proliferative response to IL-2 is controversial (34). Purified NF- κ B does not discriminate between B- and T-cell-specific sites. We believe this means that the inducible binding activity detected in both B and T cells is due to the same NF- κ B protein. This is supported by our finding that binding activity in the CEM T-cell line is stimulated by nucleoside triphosphates. It is also supported by the recent finding that a human B-lymphocyte protein similar or identical to NF- κ B activates transcription *in vitro* from the HIV LTR (35). While induction of binding represents one level of NF- κ B regulation, other events apparently distinguish the T- and B-lymphocyte response to activation.

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